abundant in the spleen, the system we have described is absent from that organ in the mouse and has so far been found only in the liver. This finding agrees with the results of tissue-slice experiments. The stability of β-glucuronidase to storage, to acetone precipitation and to ammonium sulphate fractionation is also in marked contrast with the above results. A purified spleen β-glucuronidase preparation (kindly given by Dr G. T. Mills) could not replace the liver suspension in the synthetic system. Although this result might be explicable if the system comprised more than one enzyme, we have found that saccharate and saccharic $1 \rightarrow 4$-lactone (the most powerful β-glucuronidase inhibitor known) do not interfere with the synthesis. Finally, Dr G. A. Levvy has very kindly studied the effect of a pure solution of the factor upon the hydrolysis of phenolphthalein glucuronide by mouse liver β-glucuronidase. A 4×10^{-5}M solution of the factor did not inhibit the hydrolysis of $12{\cdot}5 \times 10^{-5}$M or $6{\cdot}25 \times 10^{-5}$M substrate (within experimental error), and he concludes that the factor cannot be a substrate for β-glucuronidase. Taken together, the evidence appears to show conclusively that this enzyme is not concerned in the formation of glucuronides by the present system.

SUMMARY

1. Conditions are described for the study of glucuronide synthesis in liver suspensions.

2. Glucuronide synthesis could not be demonstrated unless a boiled extract of liver was also present. The reaction product has been shown to be a glucuronide.

3. The active factor obtained from liver by boiling or by trichloroacetic acid extraction was destroyed by acid and alkali and by phosphatase preparations.

4. The effect of pH and certain other conditions upon the synthesis has been studied.

5. The enzymic activity of the suspensions appeared to be located in the particulate material of the cell cytoplasm.

6. The synthesis in suspensions is compared with that in liver slices, and the possible relationship of β-glucuronidase to the first-named process discussed.

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Studies in Immunochemistry

14. THE ISOLATION AND PROPERTIES OF SUBSTANCES OF HUMAN ORIGIN POSSESSING BLOOD-GROUP B SPECIFICITY

By R. A. GIBBONS and W. T. J. MORGAN
The Lister Institute of Preventive Medicine, London, S.W. 1

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The isolation and properties of human blood-group substances which possess A, H and Le^a specificity from ovarian cyst fluids are described by King & Morgan (1944), Morgan & Waddell (1945), Aminoff, Morgan & Watkins (1950) and Annison & Morgan (1952*a*, *b*), and certain of the materials were shown to be substantially homogeneous both physically and chemically. The present paper describes the isolation of substances having B specificity from the same source, and records their chemical, physical and serological properties. Earlier work in this field is reviewed by Morgan (1947), Bray & Stacey (1949) and Kabat (1949).

A number of useful procedures for the purification of blood-group substances derived from ovarian cyst fluids have already been described, and it has

been found that extraction of the dried, crude cyst fluid with 90% phenol is an almost indispensible preliminary treatment. This procedure extracts the major part of the protein impurities and leaves a predominantly mucoid material, which contains most of the serological activity, as a phenol-insoluble residue. The isolation of the human A, H and Le^a substances has involved the use of 5% trichloroacetic acid at 0° (Aminoff *et al.* 1950; Annison & Morgan, 1952*a*, *b*) but the use of this reagent was avoided in the present investigation in order that the pH throughout the purification procedure could be maintained between 6 and 8, and thus avoid any possibility of degradation of the group substance due to acid or alkali.

MATERIALS AND METHODS

Materials. Ovarian cyst fluids collected as soon as possible after surgical removal of the growth, are examined serologically for their content of blood-group substances. The cyst fluids which are active and of large volume are dried from the frozen state or are stored at 0° under toluene until required. The dried cyst fluids derived from three secretors belonging to group B (patients no. 115, 137 and 192) were used as starting materials. Each cyst material was investigated separately.

Analytical methods. The methods of analysis employed are given by Annison & Morgan (1952*a*).

Serological assay. The serological activity of each fraction was determined by inhibition of agglutination of group B human erythrocytes by anti-B sera, the assay being made as described by Morgan & King (1943). Solutions of the substances under test (0·1 ml.), serum (0·1 ml.) and erythrocyte suspension (0·1 ml.) have usually been employed, but with certain test sera available in small amounts only, volumes of about 0·01 ml. have been used and the volumes of test substance and erythrocyte suspension correspondingly reduced. A 'standard' preparation of B substance was set up with each titration and the serological activity of a given sample expressed as a percentage of that of this laboratory standard. The standard used throughout was the most active crude cyst material available, and was derived from cyst no. 115. The estimated serological activity of the B active substances depends on the kind of agglutinating serum employed in the test and a preliminary report on the serological characters of anti-B sera of different origin has already been published (Gibbons & Morgan, 1952). The following sera were used to characterize serologically the most highly purified specimens of B substances. (*a*) Natural human sera from unselected individuals of blood groups A_1, A_2 and O. Eighteen different samples were examined, and they were, with one exception, found to be identical serologically under the conditions of test (see Gibbons & Morgan, 1952). Two specimens (anti-B titres 250) were used diluted 1:6 for most of the titrations. (*b*) Immune rabbit anti-B sera, obtained after immunization with an artificial antigen made from a partially purified sample of B substance and the conjugated protein component of the O somatic antigen of *Shigella dysenteriae* (Shiga) (Morgan, 1943; Rainsford & Morgan, 1946). Sera from three different rabbits were used, with entirely consistent results. (*c*) Five samples of immune human anti-B sera. Three of the sera were made by the immunization of volunteers with a partially purified sample of B substance obtained from an ovarian cyst fluid; one serum arose as a result of an incompatible transfusion, and the other through an injection of T.A.B. vaccine (Davidsohn, 1938). These immune sera behaved similarly in serological titrations. The immune sera were diluted to give an inhibition end point at a dilution of about $1:5 \times 10^5$ with the most active group B preparations.

EXPERIMENTAL AND RESULTS

The isolation of B active materials

The whole of the dried cyst material from cyst 115 (40 g.) was extracted with 400 ml. of 90% (w/w) phenol: water, the insoluble material collected by centrifuging and extracted three times with 200 ml. quantities of 90% phenol. The phenol-insoluble material was washed twice with ethanol, dissolved in water and dialysed in a cellophan bag at 0° until free from phenol. The material soluble in phenol was found to possess negligible serological activity, to contain about 14% N, and only 0·7% methylpentose; it was discarded.

The phenol-insoluble material, which contained much gelatinous material after dialysis, was made up to 1 l. in water and fractionally precipitated by the addition of acetone. The greater part of the material was precipitated at 45% (v/v) acetone concentration and was obtained as a thick gelatinous substance; this fraction contained the most active material when titrated against natural human β-serum.

The fraction precipitated between levels of 45–55% (v/v) acetone (4·7 g.; N, 5·0; methylpentose, 19·4%) was readily soluble in water to give clear, slightly viscous solutions, but possessed no greater activity than the original crude cyst material. This observation was unexpected since past experience with cyst materials of A, H and Le^a specificity had always shown that the material serologically more active was to be found in the relatively non-viscous, water-soluble materials. This fraction was further purified and the isolation from it of a homogeneous material of relatively low specific activity is described under the account of the isolation of B′ substance.

The isolation of B substance

The voluminous jelly precipitated from the aqueous solution of the phenol-insoluble fraction at 45% (v/v) acetone concentration was dialysed free from acetone and shaken mechanically with 1·5 l. of water for 3 hr. The resulting viscous solution was apparently a single phase and stable to prolonged centrifugation at 3000 *g*. The material in solution was recovered by fractional precipitation with ethanol, to which 1% (v/v) of a saturated solution of potassium acetate in ethanol had been added to assist flocculation. No precipitate formed below 33% (v/v) ethanol concentration; the largest fraction (7·6 g.; N, 6·9; methylpentose, 15·0%) was precipitated between the levels of 33 and 45% (v/v) ethanol, and was twice as active as the laboratory standard. This fraction was somewhat intractable and difficult to get into aqueous solution, the material merely swelling to give a bulky bluish grey translucent jelly. After prolonged stirring or shaking, a solution could be obtained, but the material frequently became insoluble again on standing. The addition of a small amount of 1 N-NaOH solution at 0°, followed by immediate neutraliza-

tion with acetic acid, usually resulted in a more stable solution, without apparent impairment of serological activity. This treatment was, however, avoided in view of the extreme alkali lability shown by the blood-group substances (Morgan, 1946; Knox & Morgan, unpublished observations). The material dissolved readily in neutral 50% (w/v) aqueous urea, however, and a 1% solution in this reagent was fractionally precipitated by addition of ethanol containing 1% (v/v) saturated ethanolic potassium acetate. The precipitates were washed with a little water into dialysis bags and dialysed until free from urea. The results of the fractionation are given in Table 1.

An approximately 0·5% aqueous solution of the 50–55% fraction (Table 1) was refractionated by addition of ethanol containing potassium acetate as before. No precipitate separated below 45% (v/v) ethanol level. Five fractions were collected, the yields and analyses are shown in Table 2. The two major fractions, those precipitated between 50 and 55% (v/v) ethanol concentration, were examined in the Svedberg ultracentrifuge, and it was observed that, while a considerable amount of heavy material separated rapidly shortly after reaching full speed (270000 *g*), the remainder sedimented as a single peak (Fig. 1). These two fractions (Table 2, *f* and *g*) (0·5% in 1% NaCl) were accordingly centrifuged for 1 hr. at 60000 *g* in a 'Spinco' preparative ultracentrifuge, and the supernatant fluids and deposits dialysed free from chloride ion and dried. The analyses of the two materials are shown in Table 3. The two supernatants were mixed, and examined electrophoretically. The material migrated as a single sharp peak but an early test plate exposure showed a small, rapidly moving and rapidly diffusing component which indicated contamination with a second component to the extent of a few per cent. It was evident from its mobility at pH 8 in phosphate buffer that the contaminant must carry a strong negative charge under these conditions. The material was, therefore, dissolved in phosphate buffer (pH 8), and was precipitated twice at 55% (v/v) ethanol concentration in the hope that the second

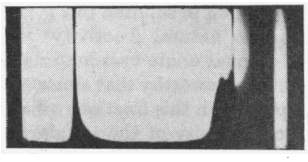

Fig. 1. Ultracentrifuge sedimentation diagram of a 1% solution of partially purified B substance in phosphate buffer, pH 8, containing NaCl; $I = 0.35$. Exposure after 20 min. at 270000 *g*.

Table 1. *Fractional precipitation of partially purified B substance* (1%, *w/v*) *from solution in* 50% *aqueous urea by addition of ethanol*

	Precipitation level (%, v/v ethanol)	Yield (g.)	Serological activity* (% of standard preparation)	N (%)	Methylpentose (%)
(*a*)	0–50	2·17	200	8·1	13·5
(*b*)	50–55	4·30	400	6·6	16·9
(*c*)	Above 55	0·05	25	10·1	7·5

* Against natural human β-agglutinin.

Table 2. *Fractional precipitation of fraction* (*b*) (*Table* 1) *from aqueous solution* (0·5%, *w/v*) *by addition of ethanol*

	Precipitation level (%, v/v ethanol)	Yield (g.)	Serological activity* (% of standard preparation)	N (%)	Methylpentose (%)
(*d*)	45–47	0·10	200	6·7	16·7
(*e*)	47–50	0·53	400	6·8	16·9
(*f*)	50–52	2·41	400	6·5	16·9
(*g*)	52–55	1·45	400	6·4	17·3
(*h*)	Above 55	0·01	—	—	—

* Against natural human β-agglutinin.

Table 3. *Ultracentrifugation of fractions* (*f*) *and* (*g*) (*Table* 2) 0·5% (*w/v*) *in* 1% (*w/v*) *aqueous* NaCl *at* 60000 *g for* 1 *hr.*

		Yield (g.)	Serological activity* (% of standard preparation)	N (%)	Methylpentose (%)
(*f*)	Supernatant	0·58	400	6·0	17·8
	Residue	1·57	400	6·5	16·5
(*g*)	Supernatant	0·38	400	5·9	17·8
	Residue	0·80	400	6·5	16·9

* Against natural human β-agglutinin.

minor component would remain in solution. Subsequent electrophoretic examination of the material failed to detect more than one component (see Addendum).

Preparations of B substances have also been obtained from cyst 192, as follows. The dried cyst material (82 g.) was extracted with 90% phenol, and a phenol-insoluble residue (about 20 g.) was obtained. This material, dissolved in 1·5 l. water, was fractionated with ethanol. No precipitate was formed at the 50% (v/v) ethanol concentration, but on the addition of 200 ml. of saturated aqueous NaCl a copious precipitate formed. The precipitate after dialysis and freeze-drying weighed 15 g. and contained 5·5% N, 14% methylpentose, and showed twice the activity of the standard B preparation measured against natural β-serum. The material remaining in solution (5·2 g.; N, 4·5; methylpentose, 12·2%; no natural β-activity) was not further examined. The original crude cyst material contained Le^b substance, and it is noteworthy that almost the whole of the Le^b activity is present in this fraction, which also possesses about 25% of the activity of the standard material with respect to immune rabbit anti-B sera. The material precipitated on the addition of NaCl to the 50% (v/v) ethanol solution was soluble in water and gave a thick gelatinous solution, similar to the gelatinous fractions obtained from cyst 115. This fraction was subjected to ultracentrifugation as described above, and the material remaining in the supernatant fluid after centrifuging for 1 hr. at 60000 *g* was further purified by successive fractional precipitations from solution in 50% (w/v) urea with ethanol, from formamide with ethanol, and from aqueous solution with Na_2SO_4. The final product contained 5·5% N, 15·6% methylpentose, 21% hexosamine, and 52% of reducing sugars; serological activity with natural serum was 400% of that of the standard. At a concentration of 1% the material sediments in the ultracentrifuge at a rate similar to that found for the B substance obtained from cyst 115, and is therefore probably of comparable particle size. Certain of its analytical figures differ by about 10% from those given by the B substance isolated from cyst 115.

The isolation of B′ substance

The fraction of the phenol-insoluble material from cyst 115 which was precipitated between levels of 45 and 55% (v/v) acetone concentrations (see above) appeared from its analysis to be almost entirely mucoid in character, but its serological activity with natural β-serum was only a quarter or less of that of the B substance, whose isolation has been described. It was originally assumed that there was present as a major contaminant an inactive mucoid material, and further fractionations were performed in an attempt to demonstrate the presence of this unspecific material and finally to eliminate it. Diethylene glycol proved to be a very selective solvent for some of the chemically similar bacterial antigens (Morgan, 1937), and the material was, therefore, extracted successively with seven portions, each of 100 ml. of this solvent. The extracts were treated with 4 times their volume of acetone and the fractions obtained examined. The results, given in Table 4, demonstrate that no significant separation of the material into fractions differing in chemical composition or serological properties was achieved. The material which had not dissolved in diethylene glycol was extracted further with 90% phenol. Annison & Morgan (1952*a*) found that an Le^a-active mucoid initially insoluble in phenol became soluble in this solvent after removal of an accompanying gelatinous material. In this instance, however, although a prolonged (2·5 days) extraction was performed, less than 1% of the material dissolved in phenol. An aqueous solution (2%, w/v) of the phenol-insoluble portion was further split into four fractions by addition of ethanol containing potassium acetate. The yields and analyses are given in Table 5. The largest fraction (Table 5; 55–57%, v/v, precipitate) appeared to be chemically and physically homogeneous (see below), but its serological activity with natural β-serum remained at the same activity as the original cyst substances. It has been found that this preparation is of high activity if immune human or animal anti-B sera are employed in the serological titration, and since it appeared to be a chemical, serological and physical entity, it has been designated B′ substance.

No material identical with B′ substance has so far been isolated from cyst fluid 192 but a number of mucoid fractions which show a greater differentiation of serological activity, in terms of reactivity with natural β-agglutinin and immune anti-B agglutinin, than material B′ have nevertheless been obtained. One material showed 3 and 100%, respectively, of the activity of the laboratory standard in titration experiments using these two anti-B reagents. The cyst fluids obtained from two 'secretors' belonging to group A_1B have also yielded mucoid preparations which show as little as 1% of the activity of the standard material when titrated against human β-agglutinin, whereas in terms of activity against immune rabbit anti-B serum the specimens were twice as active as the standard material. These mucoid materials, however, possessed only a fraction (12·5%) of the activity of the standard preparation when titrated against immune human anti-B agglutinin, and in this property were sharply distinguished from the B′ substance isolated from

Table 4. *Diethylene glycol extraction of partially purified B′ substance*

No. of extract	Yield (g.)	Serological activity* (% of standard preparation)	N (%)	Methylpentose (%)
1	0·12	100	4·9	17·8
2	0·22	100	4·7	18·4
3	0·17	100	4·9	19·2
4	0·35	100	4·8	18·9
5	0·42	100	5·2	20·0
6	0·47	100	5·2	18·9
7	0·28	100	4·8	18·2
Undissolved residue	2·92	100	5·0	19·0

* Against natural human β-agglutinin.

Table 5. *Fractionation of material undissolved in diethylene glycol (Table 4) from (1%, w/v) aqueous solution with ethanol*

Precipitation level (%, v/v ethanol)	Yield (g.)	Serological activity* (% of standard preparation)	N (%)	Methylpentose (%)
50–53	0·11	100	4·9	19·7
53–55	1·20	100	4·9	19·6
55–57	1·45	100	4·9	20·0
Above 57	0·05	100	4·9	19·6

* Against natural human β-agglutinin.

cyst 115. As anticipated, these mucoids possessed A activity. A more detailed physical and chemical examination of the mucoid fractions which possess low or negligible activity against natural β-agglutinin but are fully active when assayed against immune rabbit anti-B agglutinin, is being undertaken.

Tests for homogeneity of blood-group substances

The homogeneity of preparations of B and B′ substances obtained from cyst 115 was studied by the following procedures.

Fractional solubility test. A fractional solubility test of the type described by Aminoff *et al.* (1950) has been employed.

(*a*) *B substance.* A portion (85 mg.) of the material obtained after reprecipitation from buffer of the mucoid remaining in the ultracentrifuge supernatant fluid (Table 3) was shaken in a 50 ml. centrifuge pot with successive small portions of water. Four extracts were obtained and there remained a final residue. The yield, total N, methyl pentose, hexosamine and reducing value of each fraction are recorded in Table 6. (*b*) *B′ substance.* The material (300 mg.) was part of the 55–57 % ethanol precipitate (Table 5). It was treated in a similar manner to the B substance, but in this instance aqueous 40 % (v/v) ethanol was used as the solvent because of the ready solubility of the B′ substance in water. The yields and analyses of the five fractions obtained are recorded in Table 7.

Electrophoresis. Both preparations were examined electrophoretically at pH's 4·0 and 8·0, and no evidence of inhomogeneity was found. Details are given in the Addendum to this paper.

Ultracentrifugal examination. Both substances were examined in the ultracentrifuge. The B substance gives a sharp but asymmetric schlieren diagram, with a barely detectable amount of a heavier component. The B′ substance sediments as a single, somewhat polydisperse component. Within

Table 6. *Fractional solubility test for homogeneity of B substance*

Material derived from ultracentrifuge supernatant fluids (Table 3) was extracted with successive amounts of water.

No. of extract	Yield (mg.)	N (%) (*a*)	Methylpentose (%) (*b*)	Hexosamine* (%, as base) (*c*)	Reducing sugars* (%, as glucose) (*d*)
1	11·0	5·7	17·7	— †	— †
2	24·1	5·7	17·7	20·1	49·6
3	23·2	5·8	17·7	19·4	51·2
4	16·5	5·8	17·5	20·0	51·0
Final residue	7·2	5·8	17·9	19·5	50·5
	1st extract	2nd extract	3rd extract	4th extract	Final residue
b/*a*	3·1	3·1	3·1	3·0	3·1
c/*a*	—	3·5	3·4	3·4	3·4
d/*a*	—	8·6	8·8	8·8	8·7
c/*b*	—	1·1	1·1	1·1	1·1
d/*b*	—	2·8	2·9	2·9	2·8
d/*c*	—	2·5	2·6	2·5	2·6

* After hydrolysis in 0·5 N-HCl for 16 hr. † Lost during hydrolysis.

Table 7. *Fractional solubility test for homogeneity of B′ substance*

The 55–57 % ethanol precipitate (Table 5) was extracted with successive portions of 40 % (v/v) aqueous alcohol.

No. of extract	Yield (mg.)	N (%) (*a*)	Methylpentose (%) (*b*)	Hexosamine* (%, as base) (*c*)	Reducing sugars* (%, as glucose) (*d*)
1	27	4·8	19·9	21·3	54·4
2	40	4·8	19·8	20·3	55·1
3	75	4·9	19·7	22·1	55·0
4	108	4·9	19·8	22·2	54·3
Final residue	39	4·8	20·0	22·7	53·9
	1st extract	2nd extract	3rd extract	4th extract	Final residue
b/*a*	4·2	4·1	4·0	4·1	4·2
c/*a*	4·5	4·2	4·5	4·6	4·7
d/*a*	11·4	11·4	11·3	11·3	11·2
c/*b*	1·1	1·0	1·1	1·1	1·1
d/*b*	2·7	2·8	2·8	2·7	2·7
d/*c*	2·6	2·7	2·5	2·5	2·4

* After hydrolysis in 0·5 N-HCl for 16 hr.

the limits of the available test methods these materials are substantially homogeneous, and suitable for detailed chemical study and for comparison with the A, H, and Le^a blood-group mucoids already isolated. Details of the behaviour of the preparations in the ultracentrifuge are given in the Addendum.

The results show that on the basis of physical and chemical measurement the B and B′ substances contain little material other than the single main component.

Properties of the B substances

The properties to be described are those of the two apparently homogeneous preparations obtained from cyst 115.

Immunological properties. The B and B′ substances, although isolated from the secretions of a single individual are serologically distinguishable when examined under identical conditions. The B′ substance is found to be of the same activity as the native crude cyst material when titrated against natural human β-serum, whereas the B substance shows at least 4 times the activity. On the other hand, when titrated with human or rabbit immune sera, the B′ substance is found to be about 4 times as active as the crude starting material and somewhat more active than the B substance. The serological characteristics of the B substances are profoundly modified by heating in weakly alkaline (pH 7·7–8·0) solution in an autoclave at 120° for 30 min. and by this treatment rapidly lose their ability to inhibit natural human β-sera, whereas their capacity to inhibit animal or human immune sera is not impaired and is even somewhat enhanced in the earlier stages of the treatment (Gibbons & Morgan, 1952; see Fig. 2). After 2 hr. heating under these conditions the serological activity with immune anti-B sera begins to fall.

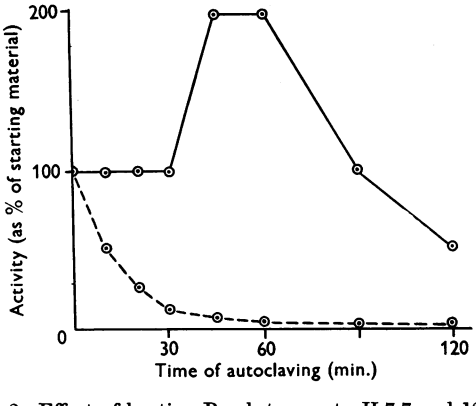

Fig. 2. Effect of heating B substance at pH 7·7 and 120°. – – –, Activity as measured by human β-serum; ——, activity as measured by rabbit immune anti-B serum.

Hydrolysis of the B and B′ substances with 1 N acetic acid at 100°, on the other hand, slowly destroys the serological activity with respect to both natural and immune sera at about the same rate; the activity falls slowly and by 16 hr. it is negligible. The B and B′ substances after hydrolysis in N acetic acid at 100° were also tested for their capacity to (i) inhibit the haemolysis of sheep erythrocytes by rabbit anti-human A red cell serum in the presence of complement; (ii) inhibit the agglutination of A_2 cells by human anti-A serum to detect that no structures which possess A specificity or 'Forssman' activity are formed during the acid treatment; (iii) precipitate with horse anti-pneumococcus type XIV serum. The products of periods of hydrolysis up to 64 hr. were studied but no evidence of the development of 'Forssman' or A activity was found. The unhydrolysed materials failed to form a specific precipitate with horse-anti-pneumococcus type XIV serum, but after hydrolysis for 16 hr. the antiserum gave a trace of precipitate with both substances at a dilution of 1:4000. After hydrolysis for 64 hr., however, the amount of precipitate formed with the antiserum at this dilution was very small, whereas the homologous type XIV polysaccharide gave heavy precipitates at all dilutions from 1:1000 to 1:100000. Materials degraded by autoclaving (see above) do not develop type XIV reactivity, but do so if subsequently hydrolysed in N acetic acid.

The B and B′ substances, when tested at a concentration of 1 mg./ml., fail to inhibit the following blood group agglutinins: (1) natural human, immune human, immune rabbit anti-A; (2) anti-M, anti-N, anti-S; (3) anti-P; and (4) the Rh antibodies anti-C, anti-D, anti-E, and anti-c. Some inhibition of anti-H and anti-Le^a agglutinins was observed, but assuming the inhibition to be due to a contamination of the preparations with these group substances and not to serological cross-reactivity, it can be calculated that not more than 1 % of H and Le^a substances are present in the B preparation. Very slight inhibition of anti-Le^b agglutinin by the B substance was observed, but the B′ substance preparation showed considerably more inhibition, equivalent to about 1 % of that of the most active preparation of Le^b substance available.

Physical properties. The B and B′ substances were examined in a Hilger quartz spectrophotometer at a concentration of 0·1 % in 0·85 % saline using an optical path of 1 cm. In the region examined (220–320 mμ.) no bands of specific absorption were found.

The viscosity of 0·5 % solutions of the two preparations in 0·85 % saline was determined in an Ostwald pattern viscometer at 37°. The viscosities relative to that of saline are, B substance, 4·1; B′ substance, 1·5. The specific rotations are, B

substance, $[\alpha]_{5461}^{20}$ 0 ± 5°; B′ substance, $[\alpha]_{5461}^{20}$ − 20 ± 5° (*c*, 1·0 in water).

Chemical properties. Solutions of the B and B′ substances (1 % each) were treated with an equal volume of the following reagents: 10 % trichloroacetic acid, 2 % phosphotungstic acid, 10 % tannic acid, 20 % sulphosalicylic acid, saturated picric acid. The B substance gave an opalescence with 10 % tannic acid, otherwise no precipitates were formed. The following colour reactions were negative for both preparations: biuret, xanthoproteic, Millon, Hopkins-Cole, Bial and Seliwanoff. A positive reaction was obtained for both preparations in the Sakaguchi test. Alkaline copper solutions (biuret test) gave rise to a heavy curdy precipitate with both materials. The elementary analyses of the two preparations are as follows: B substance: C, 41; H, 6·6; N, 5·7 % (Kjeldahl); B′ substance: C, 43; H, 7·0; N, 4·9 % (Kjeldahl). Both materials are devoid of appreciable amounts of sulphur or phosphorus. The methylpentose and acetyl contents of the two preparations are B, 17·9 and 7·0 %; B′, 20·0 and 7·1 %, respectively.

By means of the liquid-vapour partition chromatogram technique of James & Martin (1952), Dr E. F. Annison has kindly demonstrated that 96–97 % of the volatile acid obtained on acid hydrolysis of the blood group B substances is acetic acid.

The B substances after heating in 0·05 N-Na_2CO_3 at 100° and the addition of an acid solution of *p*-dimethylaminobenzaldehyde, as described by Morgan & Elson (1934) for the estimation of *N*-acetylhexosamine, give rise to a reddish purple colour and thus behave as do the other blood-group mucoids. The absorption spectrum of the chromophore is identical with that given by *N*-acetylglucosamine under the same conditions (Aminoff, Morgan & Watkins, 1952) and so presumably the reaction is due to the presence of *N*-acetylhexosamine, in a reactive condition, in the blood-group substances. The B substances, after heating for 16 min. with the 0·05 N alkali, give a colour equal to about 7 % of that given by an equal weight of *N*-acetylglucosamine after heating for its optimum time of 4 min. Owing to the difference in time at which maximum colour is developed, however, a strictly quantitative significance cannot be attached to this value. The colour/time relationship of heating curves which are obtained under these conditions may be regarded as the result of two consecutive reactions, the first the condensation of the *N*-acetylhexosamine to yield a chromogenic structure followed by the breakdown of this structure to give non-chromogenic products. In the case of the blood-group mucoids these two reactions are presumably preceded by breakdown of the mucoid to yield additional *N*-acetylhexosamine residues which possess a free aldehyde group at C-1. If it is assumed that the formation and destruction of chromogen are monomolecular reactions, and proceed with the same rate constants for the *N*-acetylglucosamine and blood-group substance, it can be deduced that the total amount of *N*-acetylhexosamine in the blood-group B substances which can react under the conditions given by Morgan & Elson (1934) is probably of the order of 14–15 %.

Hydrolysis of the B substances with acid

The B substances were hydrolysed in sealed glass ampoules at 100° with (*a*) 6 N-HCl, (*b*) 0·5 N-HCl, and (*c*) 1 N acetic acid as described by Annison & Morgan (1952*a*) for the Le^a substance.

(*a*) *Hydrolysis with* 6 N-HCl. Amino and α-amino acid nitrogen was determined on these hydrolysates, and the results are shown in Fig. 3. Amino groups are rapidly released from both B and B′ substances, a maximum value of 86 and 89 % total N, respectively, being attained after about 4 hr. α-Amino acids are released more slowly, a maximum, equivalent to 50 and 38 % total N for the B and B′ substances, respectively, being reached in about 16 hr.

(*b*) *Hydrolysis with* 0·5 N-HCl. Reducing sugars and hexosamine were determined on these hydrolysates, since the simple sugars are rapidly destroyed in 6 N-HCl at 100°. The results are shown in Fig. 4. Both analytical values reach a steady level in about 5 hr., and are equivalent to 50 and 56 % reducing sugars (as glucose), and 20 and 22 % hexosamine (as glucosamine base), for the B and B′ substances respectively. The 16 hr. 0·5 N-HCl hydrolysis products of B substance have been analysed for glucosamine and galactosamine by the method of Gardell (1953). The sample was introduced on the top of a well-washed column of Zeo-carb 225 (Permutit Co. Ltd.), and eluted with 0·3 N-HCl, 0·5 ml. fractions being collected and assayed for hexosamine. Glucosamine and galactosamine are quantitatively separated under these conditions. The glucosamine/galactosamine ratio was found to be 1·7/1, the overall recovery of the mixed hexosamines being close to 100 %. The determinations were kindly carried out by Mr C. J. M. Rondle.

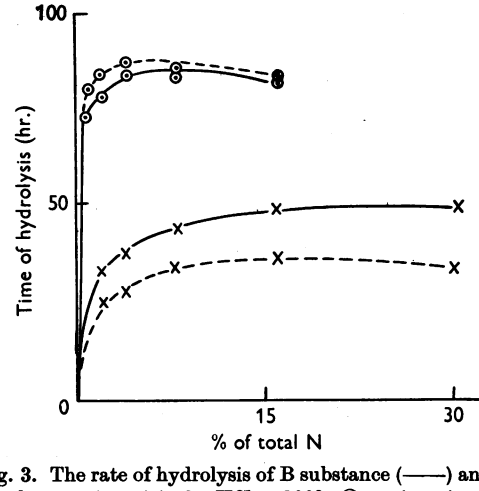

Fig. 3. The rate of hydrolysis of B substance (——) and B′ substance (– – –) in 6 N-HCl at 100°: ⊙, amino nitrogen; ×, α-amino acid nitrogen.

(c) *Hydrolysis with* N *acetic acid.* The following determinations were made on the hydrolysates. (i) Reducing sugars. There is a steady release of reducing substances amounting to about 20 and 25 % (as glucose) for the B and B′ substances, respectively, after 64 hr. (Fig. 5), but the maximum value is not reached after this time. (ii) *N*-Acetylhexosamine. The time of heating in 0·05 N-Na_2CO_3 required to give maximum *N*-acetylhexosamine colour gradually decreases from about 16 min. with the unhydrolysed B substance to 4 min. after 16 hr. hydrolysis. There is also a considerable increase in the amount of colour found for the shorter heating times e.g., after heating for 4 min. with alkali the 32 hr. hydrolysate gives a colour equivalent to 12·5 % of that given by an equal weight of *N*-acetylglucosamine, whereas the unhydrolysed mucoid only gives about 5 % of the colour after heating in alkali for this time. Periods of heating with alkali longer than 25 min., however, result in the colour given by the unhydrolysed material being greater than that given by hydrolysates. (iii) Acetaldehyde produced on oxidation with periodate. This could arise from free fucose and/or threonine, but free threonine is not found on chromatograms of mild acid hydrolysates of the B substances, whereas free fucose is readily demonstrable, and the acetaldehyde, therefore, presumably originates from fucose. It can be seen (Fig. 5) that the free fucose appears to reach a steady level after 32 hr. hydrolysis, which value (13 % for B substance and 15 % for B′ substance) is less than the total amount of fucose (18 or 20 %) as determined by the method of Dische & Shettles (1948). It is probable that the B and B′ substances, like the A substance, but unlike the H and Le^a substances, contain about a quarter of their fucose in a form stable to hydrolysis with 1 N acetic acid. (iv) Formaldehyde produced on oxidation with periodate. The determinations were carried out as described by O'Dea & Gibbons (1953). The amount of formaldehyde arising from blood-group mucoids on oxidation with periodate is a complex function of time of oxidation, due possibly to the breakdown of the polysaccharide by a mechanism similar to that suggested by Neumüller & Vasseur (1953). The amount of formaldehyde produced by the oxidation of 1 mg. of B substance, after hydrolysis in 1 N acetic acid, for four periods of oxidation, is shown in Table 8. (v) Serological examination; as given under *Immunological properties* above.

Duplicate samples (35 mg.) of B and B′ substances were hydrolysed for 4 and 16 hr., respectively, in N acetic acid. The four samples were dialysed, the diffusates stored at 0°, in the presence of $CHCl_3$, until dialysis was complete, and then both diffusible and indiffusible fractions concentrated and freeze-dried. The indiffusible fractions were analysed quantitatively, and the results are given in Table 9. The diffusible fractions were hygroscopic and could not be satisfactorily dried; their chromatographic analysis is described below. The diffusates both give a positive *N*-acetylhexosamine reaction, with a maximum colour formation after 4 min. heating with alkali which probably indicates the presence of free *N*-acetylhexosamine.

Chromatographic analysis of the acid hydrolysis products of B substances

A detailed study of the sugar and amino acid components of the B substances was made using paper chromatographic techniques. The methods and solvents used are described in detail by Annison & Morgan (1952*a*). The B and B′ sub-

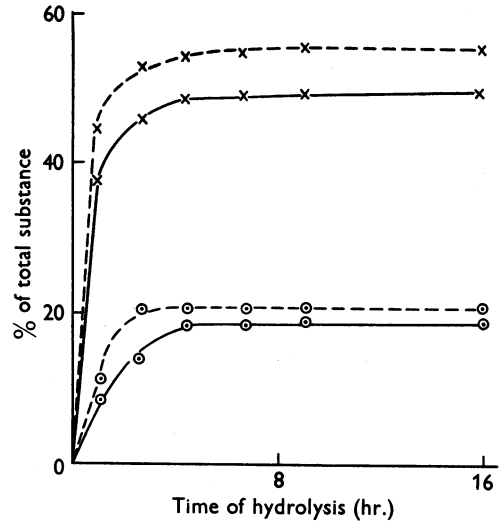

Fig. 4. The rate of hydrolysis of B substance (——) and B′ substance (– – –) in 0·5 N-HCl at 100°: ⊙, hexosamine (as glucosamine base); ×, reducing sugars (as glucose).

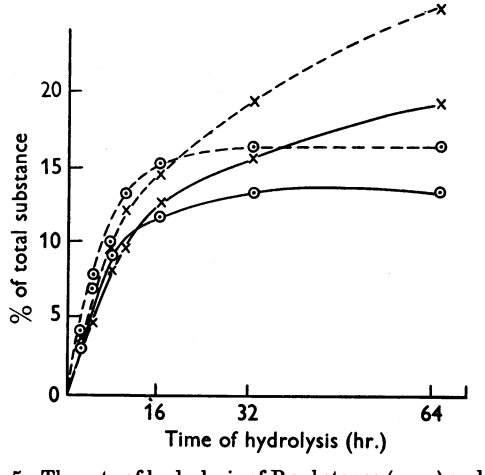

Fig. 5. The rate of hydrolysis of B substance (——) and B′ substance (– – –) in N acetic acid at 100°: ⊙, free fucose determined as acetaldehyde after periodate oxidation; ×, reducing sugars (as glucose).

Table 8. *Formaldehyde produced from* 1 *mg. of B substance after hydrolysis in* N *acetic acid and subsequent oxidation with periodate*

Time of hydrolysis (hr.)	Time of periodate oxidation (hr.) 2	4	5	24
	Formaldehyde (μg.)			
0	13·2	15·6	16·5	26·3
4	20·0	23·1	24·8	31·8
10	19·8	23·0	24·8	32·9
16	20·8	24·5	25·2	33·4
64	38·5	40·2	41·7	52·0

Table 9. *Analysis of the indiffusible portions of the B and B′ substances after hydrolysis in* N *acetic acid and dialysis*

Substance	Time of hydrolysis (hr.)	N (%)	Methylpentose (%)	Hexosamine* (%, as base)	Reducing sugars* (%, as glucose)
B	4	6·7	9·6	22·6	48·5
	16	6·3	4·6	22·6	38·0
B′	4	6·0	12·6	28·4	63·1
	16	6·4	4·8	26·8	53·1

* After further hydrolysis for 16 hr. in 0·5 N-HCl.

stances were found to be qualitatively identical with each other and with the A, H and Le^a substances.

Amino acids. Two-dimensional chromatograms of 6 N-HCl hydrolysates showed the presence of lysine, arginine, aspartic acid, glutamic acid, glycine, serine, threonine, alanine, proline, valine, and leucine (and/or isoleucine). In addition, a weak slow-moving ninhydrin-reacting spot was usually found, corresponding in position to one described by Aminoff *et al.* (1950) who attributed it to traces of cysteic acid.

Sugars and amino sugars. Galactose, fucose, glucosamine and galactosamine were identified in 0·5 N-HCl hydrolysates. In addition to the solvent systems already referred to some chromatograms were also run in mesityl oxide:30% formic acid (Bryant & Overell, 1951) for amino acids and ethyl acetate:pyridine:water (Jermyn & Isherwood, 1949) for sugars, and two-dimensional sugar chromatograms were run as described by Partridge (1948), but no additional sugar or amino acid components were detected.

The portions of each of the B substances indiffusible after hydrolysis in N acetic acid and dialysis were also examined chromatographically. Hydrolysates of these materials in 0·5 N- and 6 N-HCl gave sugar and amino acid chromatograms qualitatively similar to those given by undegraded materials but the hydrolysates of the indiffusible materials contained considerably smaller amounts of fucose (cf. Table 9). The materials which were diffusible after 4 and 16 hr. hydrolysis of each B substance with N acetic acid, when examined chromatographically without further hydrolysis with HCl showed the presence of fucose, galactose and an *N*-acetylhexosamine. Fucose was present in large amounts. In addition, a ninhydrin-reacting spot which moved very slowly, or not at all in phenol or collidine, and an unidentified substance which gave a yellow spot with hexosamine reagents (Partridge, 1948) and possessed an R_F value in collidine of about 0·23 (fucose R_F 0·44), were also found. The diffusible materials obtained from B and B′ after hydrolysis with N acetic acid were further hydrolysed in 0·5 N-HCl for 16 hr.; glucosamine, galactosamine, galactose, and fucose were detected. The yellow spot was no longer apparent.

Isolation of constituent sugars

The B substance (650 mg.) in 10 ml. of 0·5 N-HCl was hydrolysed at 100° in a sealed ampoule for 20 hr. The hydrolysate was introduced directly on to the top of a column (3·7 × 45 cm.) of Zeo-carb 225 (250- to 400-mesh), and the mixture was eluted with 0·3 N-HCl at a flow rate of about 10 ml./hr. (Gardell, 1953), fractions of 10 ml. being recovered by an automatic fraction collector. The galactose and fucose are not retained by the resin, and emerge together in the first fractions of the eluate. The glucosamine and galactosamine components are separated during their passage through the column, the former material appearing in the effluent after about 4 days, and the latter about 24 hr. later.

D-*Galactose and* L-*fucose.* The first fractions of the eluate containing the mixed sugars were pooled and Cl^- exchanged for CH_3COO^- by passage through a small (1·5 × 25 cm.) column of Amberlite IR-4 B (British Drug Houses Ltd.) in its acetate form. The solution was then concentrated *in vacuo* without risk of destruction of the sugars by strong acid.

D-*Galactose o-tolylhydrazone.* To the concentrated solution (0·5 ml.) containing the galactose and fucose was added a solution of recrystallized *o*-tolylhydrazine (200 mg., Kahlbaum) in 96% ethanol (3 ml.). The mixture was refluxed for 30 min. on a boiling-water bath, left at −10° overnight, and the crystals which formed were filtered from the mother liquor, recrystallized 3 times from hot 96% ethanol and dried over P_2O_5. The pale yellow needles (87 mg.) melted at 174°. The mixed m.p. with an authentic sample of galactose *o*-tolylhydrazone (m.p. 174°) prepared according to van der Haar (1917) remained unchanged. The optical rotation of D-galactose *o*-tolylhydrazone is small, $[\alpha]_{5461}^{20}$ being $+3 \pm 7°$ in pyridine (*c*, 1). To confirm the configuration of the galactose in the B substance therefore, the galactose *o*-tolylhydrazone isolated (67 mg.) was converted to the free sugar by heating with benzaldehyde (van der Haar, 1920). The concentrated solution was thoroughly extracted with ether and on standing at −10° with 20 times its volume of glacial acetic acid: ether (1:1, v/v) gave D-galactose (19 mg.), $[\alpha]_{5461}^{22} + 84 \pm 5°$ in water (*c*, 1).

L-*Fucose diphenylhydrazone.* The filtrate after separation of the crude galactose *o*-tolylhydrazone was treated with benzaldehyde (van der Haar, 1920) to decompose any *o*-tolylhydrazone present and remove excess of *o*-tolylhydrazine, and the aqueous solution after extraction with ether was treated with freshly distilled 1:1-diphenylhydrazine (150 mg.) in 96% ethanol (2 ml.) and ethanol was added until a clear solution was obtained. The solution was kept at 37° overnight, cooled to −10°, and the crystals which separated were filtered from the mother liquor, dissolved in the minimum amount of hot 96% ethanol and the solution poured into a large volume of ether. The precipitate formed was filtered off, recrystalized 3 times from 96% ethanol and dried over P_2O_5, giving 27 mg. of fine white needles. The fucose diphenylhydrazone had $[\alpha]_D^{22} - 14 \pm 5°$ in pyridine (*c*, 1) and m.p. 199°, unchanged by admixture with an authentic sample prepared as described by Muther & Tollens (1904).

D-*Glucosamine hydrochloride.* The fractions issuing from the column which contained glucosamine were pooled, concentrated to a small volume, treated with an excess of

acetone and kept at $-10°$ for 3 days. The crystals which formed were filtered off, washed with cold acetone and ether, and dried over P_2O_5. The D-glucosamine hydrochloride (yield, 50 mg.) had $[\alpha]_{5461}^{22} + 73 \pm 5°$ in water (*c*, 1). (Found: N, 6·6; Cl^-, 17·2. Calc. for $C_6H_{14}O_5NCl$: N, 6·50; Cl^-, 16·5%.) Assayed for hexosamine by the method of Elson & Morgan (1933) and by the method of Smithies (1953), the specimen gave 99–100% of the colour intensity given by an authentic specimen of D-glucosamine hydrochloride.

D-*Galactosamine hydrochloride*. The fractions issuing from the column which were known to contain galactosamine were treated in the same manner as those which contained glucosamine, yielding 19 mg. of white microcrystalline powder. The D-galactosamine hydrochloride showed $[\alpha]_{5461}^{22} + 92 \pm 5°$ in water (*c*, 1). (Found: N, 6·5; Cl^-, 17·0. Calc. for $C_6H_{14}O_5NCl$: N, 6·5; Cl^-, 16·5%.) A hexosamine determination on the material by the methods of Elson & Morgan (1933) and Smithies (1953) gave 98·7 and 25·0%, respectively, of the colour intensity of an equal weight of an authentic specimen of glucosamine hydrochloride. The method of Smithies (1953) involves acetylation of the hexosamine and subsequent measurement of the *N*-acetylhexosamine. *N*-Acetylgalactosamine, however, is known to give only 23% of the colour intensity given by an equal weight of *N*-acetylglucosamine under the conditions described by Morgan & Elson (1934) and Aminoff *et al.* (1952) and, therefore, the results may be considered as offering additional evidence for the identity of the hexosamine as galactosamine. All m.p.'s quoted are uncorrected.

DISCUSSION

The isolation of substances possessing blood-group B activity from ovarian cyst fluids has enabled these materials to be characterized as apparently homogeneous molecular species and compared with the A, H and Le^a substances of similar purity already described. Two interesting and probably important observations can be recorded. First, it has been found possible to isolate from the secretion of a single individual two chemically and physically distinct mucoids, designated B and B′ substances, each of which possesses a characteristic reactivity with anti-B agglutinins of different origin. Kauertz (1938) reported that the saliva of certain group B secretors contains two serologically distinguishable B substances, but we believe that the difference between B and B′ substance is not related to the differences reported by him; nor to the differences between the partial receptors B_1, B_2, B_3 which were identified in the human B red cell antigen by Friedenreich & With (1933). The significance of our observation is not altogether clear, but there arise interesting possibilities which will be considered. Secondly, the B substance as isolated from two cyst fluids (nos. 115 and 192) has a particle weight of the order of $1·8 \times 10^6$, whereas the weight of the other blood-group substances, already reported, are considerably less and fall within the range $2·6–3·1 \times 10^5$.

The B substance has been isolated from a cyst material which was gelatinous and soluble in water with difficulty. Material with these properties has in the past not been extensively investigated, and it is probable that active materials with a particle weight comparable with that of the B substance could also be isolated from suitable cyst fluids which possess A, H or Le^a specificity. The B′ substance, however, has been obtained from a fraction of cyst mucin corresponding to that from which the A, H and Le^a substances were isolated and the final material obtained is physically very similar to them. The B substance is very viscous in aqueous solution, whereas the A, H and Le^a substances so far described yield solutions of low viscosity except at very high concentrations. The B substance appears to be without action on polarized light, whilst the B′ substance possesses a rotation of about $-20°$. Qualitative chemical examination reveals no difference between the B and B′ substances and the A, H and Le^a substances; all contain L-fucose, D-galactose, D-glucosamine and D-galactosamine, and the same 11 amino acids. Quantitatively, however, the B and B′ substances differ significantly from the A, H and Le^a substances in that they contain only 20 and 22% hexosamine, respectively, as against 33–37% for the other blood-group substances.

A number of preparations isolated from the cyst fluids of secretors belonging to group B have been obtained in this laboratory during the past 6 years. These preparations have not always single peaks on electrophoresis, but in every instance the hexosamine content was found to lie between 20 and 25%. Comparable materials from group A cyst fluids have been found to possess a hexosamine content greater than 33%, and it seems highly probable that the products of genes *A* and *B* found in ovarian cyst fluids differ considerably in their content of amino sugar. The results of a detailed study of the ratio of glucosamine to galactosamine in these materials will be given later.

Baer, Kabat & Knaub (1950) report that the B substances isolated by them from autolysed horse stomachs are low in hexosamine, although the materials obtained from human group B saliva by these authors give hexosamine figures which fall within the same range (19–30%) as those given by materials obtained from the saliva of individuals of groups A and O.

About the same amounts of reducing substances are liberated from all the blood-group substances on hydrolysis with 0·5N-HCl. The B substances must, therefore, contain correspondingly larger amounts of non-hexosamine reducing sugar, which can only be galactose since the fucose content is known and is about the same as that of the A substance. From the reducing value obtained on hydrolysis of the B substances, it can be calculated that about 30%

galactose is present, but an alternative method for the direct estimation of galactose in the presence of other sugars and amino acids is very desirable. The B substance also appears to contain somewhat more amino acid residues in its molecule (about 30%) than do the other blood-group substances (about 22%). The B and B′ substances behave in a qualitatively similar manner to those of the other group materials on hydrolysis with N acetic acid; all yield fucose as free sugar. The B and B′ substances behave like the A substance in that only some three-quarters of the total fucose is split off under these conditions, whereas the preparations of H and Le^a substances so far investigated appear to contain no acid-stable fucose. Preparations of H substance, however, containing more than 14% fucose have recently been obtained but no examination for the presence of acid-stable fucose has yet been made. In addition to fucose, small amounts of free galactose and *N*-acetylhexosamine are also detached from the B substances by N acetic acid at 100° but apart from fucose, the only identifiable free sugar liberated from A substance under these conditions is an *N*-acetylhexosamine. A ninhydrin-reacting substance which fails to give a positive reaction with Ehrlich reagents and which is diffusible through a cellophan membrane is also detectable in the N acetic acid hydrolysis products of the blood-group substances and the B and B′ substances likewise yield a similar material, but in view of the increase in nitrogen content of the indiffusible portions of the mucoids after hydrolysis and dialysis it seems that mild acid hydrolysis is associated almost exclusively with degradation of the polysaccharide part of the molecule.

The oxidation with periodate of the N acetic acid hydrolysis products of B substance indicates that for any given period of oxidation the amount of formaldehyde found rises rapidly during the first 4 hr. of hydrolysis and then remains essentially unchanged for the following 12 hr., whereas the free-reducing groups and free fucose are liberated at a steady rate throughout the whole course of the hydrolysis. The formaldehyde produced by oxidation of the B substance with periodate could arise from hexoses having C-5 and C-6 unsubstituted, i.e. hexoses possessing a free reducing group or those glycosidically linked hexoses present in the furanose form. It could also arise from free serine, but it is known that the amino acid portion of the substance is relatively stable to hydrolysis in N acetic acid and the contribution of serine is likely to be small. The significance of the formaldehyde generated cannot be unequivocally interpreted at present, since the course of periodate oxidation of polysaccharides is not yet fully elucidated. The results of a study of the formation of formaldehyde on oxidation of blood-group substances and their degradation products with periodate will, however, be reported later.

The statement has been made in the past that the blood-group substances give an enhanced *N*-acetylhexosamine colour in the early stages of hydrolysis with N acetic acid (Aminoff *et al.* 1950; Annison & Morgan, 1952*a*, *b*). This conclusion was based on the development of the colour after heating the mucoids in 0·05N sodium carbonate for what was believed to be the optimum time, i.e. about 15 min. The time of heating in alkali necessary to develop the maximum amount of chromogen is, however, altered during hydrolysis with weak acid and the amount of colour developed after 4 min. heating in alkali under the standard conditions increases throughout the period of acid hydrolysis up to 48 hr. The amount of colour developed after 15 min. heating in alkali increases during the early stages of acid hydrolysis and then decreases, whereas the colour intensity obtained after heating for 30 min. with dilute alkali decreases throughout the period of hydrolysis with acid. It appears, therefore, that mild acid hydrolysis renders the *N*-acetylhexosamine residues in the mucoid more susceptible to the action of 0·05N sodium carbonate, but, as yet, it cannot be readily determined whether or not the total amount of reactive *N*-acetylhexosamine is increased.

It is considered unlikely that the B′ substance is an artifact arising from the method of preparation since it has been found that the B substance is not demonstrably altered and changed into B′ substance under any of the conditions to which it has been subjected during isolation. It is, however, possible to degrade the B substance by heating at pH 7·7 so that it is modified and resembles the B′ substance in serological behaviour but not in chemical properties. It is possible that changes in the mucoid components of the mucin can be brought about after secretion by the tissue cells as a result of enzymic action or owing to the lability of the mucoid structure at body temperature and pH.

All persons produce and secrete mucin, and these secretions are probably essential for the normal working of the body and for the life of the individual. The results of immunogenetical and chemical studies have established that the blood-group genes play an important part in directing the synthesis of many, if not all, of the neutral mucoid materials produced by the body, whether as water-soluble secretions or as surface components of tissue cells. It seems probable that the synthetic mechanism which normally produces certain fundamental mucoid materials, which are common to all persons irrespective of their ABO grouping, is modified under the influence of the individual blood-group genes. Thus, for example, in the presence of the blood-group *A* gene the individual mucoids of the mucin formed are in part given a composition and configuration which results in their possessing an

immunological behaviour recognized as group A character. If the *B* gene is also present in the genotype some of the mucoid complexes produced possess group B serological character. Indeed, according to the genotype of the individual, the mucoids formed possess A, B, Le^a, Le^b and H serological character and the erythrocytes show the same or closely similar immunological properties.

This concept of the mode of action of genes is not new. In the field of the human blood groups Friedenreich, Thyssen & Hartmann (1939) proposed a similar scheme to account for the observed serological differences between the A character of different secretions and tissue cells. Wiener & Wexler (1952) likewise consider that the blood-group genes bring about a modification of mucopolysaccharides which are common to all human beings.

The modification brought about by genes *A*, *B*, Le^a or Le^b is characteristic of the gene's activity and results in the formation of materials which, although conforming to basic molecular patterns, nevertheless are different chemically and, as a result, show a sharply specific and characteristic immunological behaviour. The specimens of B substance so far examined contain considerably less hexosamine and more galactose than specimens of A substance, and it is believed that the chemical changes induced in the mucoid materials under the influence of the blood-group genes are larger and more definite than was originally supposed. Nevertheless, it seems that these changes, important genetically and immunologically, are of little consequence from the point of view of the general physiological function of mucins and indeed there appears to be no practical difference in the survival rate of secretor or non-secretor persons belonging to the groups A, B or O.

The result of a detailed examination of the mucoid materials produced by group B individuals indicates that there is also a variation in the properties of the B substances produced. The essentially homogeneous substances B and B′ isolated from cyst fluid no. 115 both show group B character and yet possess different physical, chemical and serological properties. Similarly, the B group materials isolated from persons belonging to A_1B possess group B serological specificities which allow them to be differentiated from either B or B′ substances.

Thus is appears probable that mucin derived from a person belonging to group A or B will not contain a single substance which possesses either A or B serological character, but a number of mucoids each of which have a right to the designation A substance or B substance as the case may be. Kabat, Baer & Knaub (1949) put forward similar ideas, but were not able to exclude the possibility that the differences in chemical composition they found did not arise as a result of the method of isolation or were due to the inhomogeneous nature of the products studied. Both these possibilities for the differences recognized between B and B′ substances can be excluded, although it is not possible to be certain that B′ substance has not been derived from B substance by degradation *in vivo* or is a product of a partial or distorted synthesis, the result of an imperfect synthesizing mechanism.

Evidence which will decide in favour of one or more of these hypotheses cannot readily be obtained, and probably the most useful immediate approach to the problem will be to undertake a detailed examination of a large number of highly purified and accurately characterized blood-group substances for some correlation between a particular chemical property and a serological specificity.

SUMMARY

1. Two purified mucoid substances, designated B and B′, which possess blood-group B character have been isolated from human ovarian cyst fluids.

2. The B substance inhibits the agglutinating action of natural human β-agglutinin or immune anti-B agglutinin on group B red cells with about equal facility, whereas the B′ substance has a considerably smaller power to neutralize the β-agglutinin but a greater capacity to prevent haemagglutination with immune B antibody. The B activity of mucoids isolated from two group A_1B cyst fluids showed a negligible capacity to inhibit natural β-agglutinin but were nevertheless fully active when assayed against immune rabbit anti-B sera.

3. The presence of L-fucose, D-galactose, D-glucosamine and D-galactosamine in the B and B′ substances has been established. Eleven amino acids have also been tentatively identified in these substances.

4. The acid hydrolysis products of B and B′ substances contain respectively, 17·9 and 20% fucose, 50 and 56% reducing substances, expressed as glucose, and 20 and 22% hexosamine. The α-amino acid nitrogen is equivalent to 50 and 38% and the amino nitrogen to 86 and 89% of the total nitrogen, respectively.

5. Possible relationships between the B and B′ substances are considered and the possibility is discussed that there exist several products of the action of a single blood-group gene.

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ADDENDUM

Physico-chemical Examination of Human Blood-Group B Substances

By E. A. CASPARY
The Lister Institute of Preventive Medicine, London, S.W. 1

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The blood-group B substances were examined in the electrophoresis apparatus and ultracentrifuge, in addition the diffusion constants and partial specific volumes were determined.

METHODS

Electrophoresis. The samples were examined in a Tiselius electrophoresis apparatus at 0°, using the diagonal schlieren optical system and monochromatic light, $\lambda = 546$ mμ. Solutions (1·5–2·0%, w/v) were made in suitable buffers and dialysed at 2° for at least 24 hr. The concentrations were chosen in order to facilitate the detection of traces of protein contaminants.

Sedimentation. Ultracentrifugal examination was carried out in a Svedberg oil turbine machine at 60000 rev./min. (270000 *g*) using a diagonal schlieren optical system (Philpot, 1938). Solutions were made in phosphate buffer, pH 8, $I = 0{\cdot}2$, with added NaCl increasing the ionic strength to 0·35 to depress charge effects on sedimentation.

Diffusion. The diffusion measurements were carried out by the Gouy interferometric method (Kegeles & Gosting, 1947) in a modification of the apparatus of Gosting, Hanson, Kegeles & Morris (1949) at $25 \pm 0{\cdot}01°$. Increased sensitivity was obtained by using the blue line $\lambda = 436$ mμ. isolated from a high-pressure mercury arc. Boundaries were formed by flowing through a capillary (Kahn & Polson, 1947).

Partial specific volumes. These were determined pycnometrically at 25°, the materials being dissolved in the buffer used for the sedimentation measurements.

RESULTS

The B′ substance showed only one component after prolonged electrophoresis in both phosphate buffer, pH 8, $I = 0{\cdot}2$, and acetate buffer, pH 4, $I = 0{\cdot}2$. At pH 8 the ascending boundary was symmetrical, the descending side gave a skewed appearance (Fig. 1). At pH 4 both boundaries were skewed and considerably spread. Migration was anodic in both cases, but slower at pH 4.

The B substance gave a single sharp component at pH's 4 and 8 under comparable conditions. Both boundaries showed a slight asymmetry, migration being anodic though again slower at pH 4.

In the ultracentrifuge at pH 8, $I = 0{\cdot}35$, B′ substance showed a single, almost symmetrical, component, remaining relatively sharp even after 60 min. at 60 000 rev./min. (270 000 g) in a 1 % (w/v) solution. The sedimentation constants showed a linear concentration dependence over the range examined (0·5–1·0 %, w/v).

The B substance under the same experimental conditions gave one very sharp major component, together with a very small amount of heavy material. The main component retained its sharpness even after 80 min. at 60000 rev./min. (270000 g) in 1 % (w/v) solution. The sedimentation constants showed a rapid increase with dilution over the range examined (1·0–0·25 %, w/v).

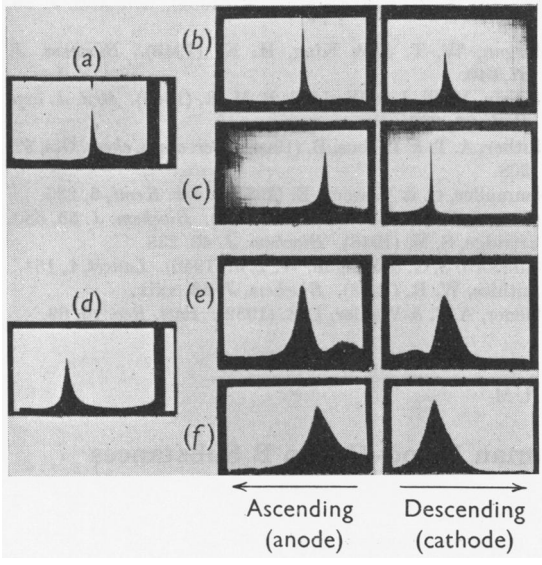

Fig. 1. Sedimentation diagrams and electrophoresis patterns of blood-group B substances. B substance, (*a*) sedimentation after 70 min. at 270000 g, 1·0 % (w/v) in phosphate: NaCl (pH 8, $I = 0{\cdot}35$). (*b*) Electrophoresis pattern after 53·5 mA-hr. of 1·5 % (w/v) solution in phosphate, pH 8, $I = 0{\cdot}2$. (*c*) Electrophoresis pattern after 45 mA-hr. of 1·5 % (w/v) solution in acetate, pH 4, $I = 0{\cdot}1$. B′ substance, (*d*) sedimentation after 40 min. at 270 000 g, 1·0 % (w/v) in phosphate: NaCl. (*e*) Electrophoresis pattern after 46 mA-hr. of 2·0 % (w/v) solution at pH 8. (*f*) Electrophoresis pattern after 50 mA-hr. of 2·0 % (w/v) solution at pH 4. Lower arrows show direction for all electrophoresis patterns.

Diffusion constants for both B substances were measured in phosphate:NaCl, pH 8, $I = 0{\cdot}35$, at 25°. The difficulty of boundary sharpening using these extremely viscous solutions and the high loss in light transmission due to scatter necessitated the use of low concentrations of material, especially with B substance. Calculations were made from the outermost fringe in all cases, approximating to D_A as defined by Grålen (1941). In both cases considerable deviation from the ideal form as defined by the probability integral, was indicated.

The partial specific volumes found were 0·604 for B′ and 0·600 for B substance.

Molecular weights were calculated from the formula of Svedberg (Svedberg & Pedersen, 1940) by combining sedimentation and diffusion constant values. The value used for the sedimentation constant was obtained by extrapolation to a concentration corresponding to that at which the diffusion had been determined. Using the appropriate values for the partial specific volumes the molecular weights found were 460000 for B′ and 1800000 for B substance, the corresponding frictional ratios f/f_0 were 3·9 and 5·7, respectively.

DISCUSSION

The data show that electrophoretically both B substances are single components, that is, the nitrogen of these substances is electrophoretically inseparable from the carbohydrate and not attributable to protein contaminants. The broad boundaries obtained with B′ indicate some polydispersity, this effect being masked in the case of B substance by its extremely high viscosity and low diffusion coefficient.

From the appearance of the ultracentrifugal boundary B′ substance appears homogeneous. The B substance shows a small, probably polydisperse, heavy component. Sedimentation constants refer to the movement of the mode of the main sedimenting boundary in each case. Both materials show non-ideal diffusion, these deviations can be

Table 1. *Sedimentation and diffusion constants of the blood-group B substances*

Values corrected to water at 20° (Svedberg & Pedersen, 1940).

B′ substance			B substance		
Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^{7}$ (corr.)	Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^{7}$ (corr.)
1·00	6·25	—	1·00	7·24	—
0·75	7·22	—	0·75	8·87	—
0·50	8·27	—	0·50	11·89	—
0·40	—	1·14	0·25	14·71	—
0·23	—	1·25	0·22	—	0·51
			0·11	—	0·56

attributed to three causes, namely, non-ideal boundary formation, concentration dependence of the diffusion constant and polydispersity, all factors being operative in this case. In the case of B substance it was found experimentally impossible to calculate the coefficient for the main component only, the diffusion constant determined represents an average as defined by Quensel (1942). The calculated molecular weight of B substance stands therefore only as an approximation. Both B substances are of greater molecular size and asymmetry than the other blood-group substances (Kekwick, 1950, 1952*a*, *b*), but very similar from the standpoint of comparative homogeneity. The B substances both show considerable asymmetry with axial ratios of 200 and 100, respectively, or alternatively may be highly hydrated.

SUMMARY

1. The B substances have been shown to give single peaks in an electrophoresis apparatus at pH's 8 and 4.

2. The molecular weight calculated from sedimentation and diffusion data is 460000 for B′ and approximately 1800000 for B substance, with frictional ratios 3·9 and 5·7, respectively.

3. The B substances are both somewhat polydisperse and probably highly asymmetrical in shape.

I wish to acknowledge the technical assistance of Mr H. Murray.

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The Branched-chain Fatty Acids of Butterfat

4. THE ISOLATION OF (+)-12-METHYLTETRADECANOIC ACID AND OF 13-METHYLTETRADECANOIC ACID

By R. P. HANSEN, F. B. SHORLAND and N. JUNE COOKE
Fats Research Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

(*Received* 30 *November* 1953)

Branched-chain fatty acids have been shown to be present in trace quantities in butterfat (Hansen & Shorland, 1951*a*, *b*, 1953; Hansen, Shorland & Cooke, 1951), in ox fat (Hansen, Shorland & Cooke, 1952*a*), in mutton fat (Hansen, Shorland & Cooke, 1952*b*, 1953), and in shark liver oil (Morice & Shorland, 1953). Two isomeric C_{15} branched-chain acids, namely (+)-12-methyltetradecanoic acid and 13-methyltetradecanoic acid, which were reported as occurring in mutton fat (Hansen *et al.* 1953), have now been isolated from butterfat.

EXPERIMENTAL

The butterfat used in this investigation was prepared by washing and centrifuging fresh butter supplied by the Rangitaiki Plains Dairy Co. Ltd., Whakatane, New Zealand. The butter had been churned in the first week of May 1951, from a bulked sample of cream representative of numerous dairy herds in the district.

Butterfat glycerides (sample G/43: 17·85 kg.; sap. equiv. 250·0; iodine value 40·1) were hydrogenated at 180° and at atmospheric pressure using Ni supported on kieselguhr as catalyst. The resulting glycerides (iodine value 1·1) were saponified in the cold with KOH and the soaps converted into fatty acids with H_2SO_4. Steam distillation removed part of the steam-volatile acids, after which the remaining mixed acids were washed with water and then repeatedly crystallized from 10 vol. acetone at −33°. The resulting 'liquid' acids were converted into methyl esters (1876 g.; sap. equiv. 223·8; iodine value 4·7) and were fractionated *in vacuo* in a 490 × 3·8 cm. stainless steel column packed with 3–4 mm. diameter single-turn glass helices.

Of the fractions distilled over, the 32nd (KL32; 48·00 g.; sap. equiv. 275·6; iodine value 7·9) was selected for the investigation reported in this paper. When freed of unsaponifiable matter this methyl ester fraction had sap. equiv. 250·4; iodine value 0·9; m.p. −4·1 to −2·0°. Fractional distillation of KL32 was then repeated at 0·05 mm. in a 50 × 1·8 cm. column fitted with a closely coiled spring as packing (column *E*, Shorland, 1952). Results of fractionation are shown in Table 1.